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In re Application of:
Canne, L., et al.

Serial No.: To be Provided

Filed: Herewith

Divisional of U.S. Patent Appln. Ser. No.: 09/097,094
(Filed on June 12, 1998)

For: **Solid Phase Native Chemical Ligation Of
Unprotected Or N-Terminal Cysteine
Protected Peptides In Aqueous Solution**

Examiner:

Art Unit:

Atty Dkt. No.: 03504.283B

PRELIMINARY AMENDMENT

Hon. Director of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Prior to commencing the examination of the above-described patent application, Applicants respectfully request entry and consideration of the following Preliminary Amendments.

Amendments

In the Specification:

Please replace pages 1, 14, 15, 32, 34, 36, 38, and 42 and Figure 30 of the Specification with the enclosed replacement pages (Appendix C). The specific nature of the changes made to these pages is shown in Appendix A.

Please incorporate the enclosed SEQUENCE LISTINGS (Appendix C) after page 49 of the Specification.

In the Claims:

Please cancel claims 1-22 and 27-28.

Please amend claims 23-26 to read as follows:

- 23. **[Amended]** A kit for preparing assembled polypeptides comprising:
- (a) a first container containing a solid support, having bound thereto a first partially or completely unprotected peptide having an N-terminus and a thioacid or a thioester of the formula $-\text{COSR}$ at its C-terminus and, wherein said partially or completely unprotected peptide is bound to said solid support via a linker; wherein said linker comprises a cleavable moiety and said partially or completely unprotected first peptide segment is bound to said linker at said N-terminus, and wherein R is a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof;
 - (b) a second container containing a set of second partially or completely unprotected peptides, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said second peptide segment is capable of selectively ligating to the C-terminus of said solid phase-bound first peptide to form a solid phase-bound peptide comprising a thioacid at its C-terminus; and
 - (c) one or more additional containers each containing a set of different partially or completely unprotected peptide segments, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each of the members of each set have the same number of amino acids.
24. **[Amended]** The kit of claim 23, wherein said set of second unprotected peptide segments (b) is comprised of peptides having the same length, but different amino acid sequences.

25. **[Amended]** The kit of claim 23, wherein said set of second unprotected peptides (b) consists essentially of identical peptides.
26. **[Amended]** The kit of claim 23, wherein said one or more sets of different unprotected peptides (c) comprise at least one set of peptides having the same length but different amino acid sequences. --

Please add the following new claims:

- 29. **[New]** The kit of claim 23, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.
30. **[New]** The kit of claim 23, wherein the C-terminus of said first partially or completely unprotected peptide comprises said thioester of formula COSR.
31. **[New]** The kit of claim 23, wherein the C-terminus of said second partially or completely unprotected peptide comprises a thioacid.
32. **[New]** The kit of claim 23, wherein the C-terminus of said second partially or completely unprotected peptide comprises said thioester of formula COSR.
33. **[New]** The kit of claim 23, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.
34. **[New]** The kit of claim 23, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.
35. **[New]** The kit of claim 23, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.
36. **[New]** The kit of claim 23, wherein said solid phase is a bead resin.

37. **[New]** The kit of claim 23, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.
38. **[New]** The kit of claim 23, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.
39. **[New]** The kit of claim 23, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.
40. **[New]** The kit of claim 23, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.
41. **[New]** A kit for preparing assembled polypeptides comprising:
 - a) a solid phase support having bound thereto a partially or completely unprotected first peptide segment comprising an N-terminus and a C-terminus, wherein said peptide is bound to said support via a cleavable linkage between C-terminus and said support, and wherein said N-terminus is a cysteine residue.
 - b) a set of second partially or completely unprotected peptides, each comprising a thioester of formula COSR or a thioacid at its C-terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said solid phase-bound first peptide segment is capable of selectively ligating to the C-terminus of said second peptide to form a solid phase-bound peptide comprising a cysteine at its N-terminus; wherein R is a straight or branched C₁₋₁₅ functionalized alkyl group, a C₁₋₁₅ aromatic structure, or 1 to 4 amino acids or derivatives thereof; and
 - c) one or more sets of different partially or completely unprotected peptide segments, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each

of the members of each set have the same number of amino acids.

42. **[New]** The kit of claim 41, wherein said set of second unprotected peptide segments is comprised of peptides having the same length, but different amino acid sequences.
43. **[New]** The kit of claim 41, wherein said set of second unprotected peptides consists essentially of identical peptides.
44. **[New]** The kit of claim 41, wherein said one or more sets of different unprotected peptides (c) comprise at least one set of peptides having the same length but different amino acid sequences.
45. **[New]** The kit of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.
46. **[New]** The kit of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises said thioester of formula COSR.
47. **[New]** The kit of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises a thioacid.
48. **[New]** The kit of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises said thioester of formula COSR.
49. **[New]** The kit of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.
50. **[New]** The kit of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.

51. **[New]** The kit of claim 41, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.
52. **[New]** The kit of claim 41, wherein said solid phase is a bead resin.
53. **[New]** The kit of claim 41, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.
54. **[New]** The kit of claim 41, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.
55. **[New]** The kit of claim 41, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.
56. **[New]** The kit of claim 41, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure. --

Remarks

Status

Applicants have cancelled claims 1-22 and 27-28. Accordingly, Claims 23-26 are pending and new Claims 29-56 have been added.

Applicants have amended claims to more clearly describe Applicants' invention. The present invention pertains to a kit for preparing assembled polypeptides. Support for the recitation of "partially or completely unprotected peptide" can be found at page 5, lines 3-5. Applicants have additionally amended the claims to recite the use of "a thioacid or thioester of the formula -COSR." Support for this recitation can be found on page 6, lines 17-18 and page 7, lines 1-2. The claims have additionally been amended to recite the formula -COSR, wherein R is "a straight or branched C₁₋₁₅ functionalized alkyl group, a C₁₋₁₅ aromatic structure, or 1 to 4 amino acids or derivatives thereof." Support for this recitation can be found on page 18, lines 8-11.

The claims have additionally been amended to recite a second peptide segment that is “capable of selectively ligating to the C-terminus of said solid phase-bound first peptide to form a solid phase-bound peptide comprising a thioacid at its C-terminus.” Support for this recitation can be found on page 6, lines 18-24.

Applicants have additionally added new claims to recite a partially or completely unprotected peptide comprising “a thioester of the formula COSR” or “a thioacid.” Support for this recitation can be found on page 6, lines 15-20 and page 18, lines 7-13. Applicants have additionally added claims to recite a polypeptide of “20 to 1000 amino acids in length.” Support for this recitation can be found on page 16, line 30. Support for the recitation of the solid phase being a bead resin can be found on page 17, line 20. Support for the recitation of a peptide segment of “5 to 99 amino acids in length” can be found on page 17, line 1. Support for the recitation of a peptide segment prepared by “solid phase synthesis” can be found on page 17, line 5. Support for the recitation of a “last peptide segment derived from recombinant DNA expression” can be found on page 17, line 7. Support for the recitation of a peptide segment with “an unnatural backbone structure” can be found on page 17, line 3. The remaining amendments to the claims have been made solely to remedy minor informalities or to address typographical or formatting errors.

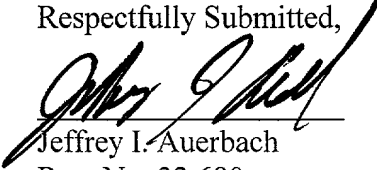
The specification has been amended to recite that the present application is a divisional of United States Patent Application Serial no. 09/097,094. In addition, pages 14 and 15 have been amended to conform to the Figures submitted with the original application. As will be recognized, original Figures 26, 28, and 29 do not contain Figure A and Figure B components. The text of the specification has accordingly been amended to conform to the original Figures. On page 15, the specification referred to Figure 32(A-H). As will again be recognized, this is a typographical error; the text is referring to original Figure 30. Since original Figure 30 does not contain A-H components, the figure has been amended to add reference to such components.

No new matter has been introduced by any of the requested amendments. Applicants respectfully submit that the present application is in condition for

Examination, and earnestly solicit early notice of favorable action. The Examiner is invited to contact the undersigned regarding any issue in this case.

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Respectfully Submitted,


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11/15/2001 11:15:00

Appendix A: The Nature of the Requested Amendments

To facilitate the Examiner's review of the patentability of the present invention, Applicant has reproduced below the specific nature of the requested amendments.

In the Specification:

Page 1 has been amended as follows:

**-- SOLID PHASE NATIVE CHEMICAL LIGATION
OF UNPROTECTED OR N-TERMINAL CYSTEINE PROTECTED PEPTIDES
IN AQUEOUS SOLUTION**

CROSS-REFERENCE TO RELATED APPLICATIONS

This application **is a continuation-in part of, and** claims the benefit of, U.S. Provisional Application No. 60/049,553, filed June 13, 1997, **herein incorporated by reference, and is a divisional application of, and claims the benefit of, U.S. Patent Application Serial No. 09/097,094, herein incorporated by reference.**

Background

Existing methods for, the chemical synthesis of proteins include stepwise solid phase synthesis, and fragment condensation either in solution or on solid phase. The classic stepwise solid phase synthesis of Merrifield involves covalently linking an amino acid corresponding to the carboxy-terminal amino acid of the desired peptide chain to a solid support and extending the polypeptide chain toward the amino end by stepwise coupling of activated amino acid derivatives having activated carboxyl groups. After completion of the assembly of the fully protected solid phase bound peptide chain, the peptide-solid phase covalent attachment is cleaved by suitable chemistry and the protecting groups removed to give the product polypeptide.

Some disadvantages of the stepwise solid phase synthesis method include: incomplete reaction at the coupling and deprotection steps in each cycle results in formation of solid-phase bound by products. Similarly, side reactions due to imperfections in the chemistry, and or impurities present in the reagents/protected amino acids, all lead to a multiplicity of solid phase bound products at each step of the chain assembly and to the formation of complex product mixtures in the final product. Thus, the longer the peptide chain, the more challenging it is to obtain high-purity well-defined

products. Due to the production of complex mixtures, the stepwise solid phase synthesis approach has size limitations. In general, well-defined polypeptides of 100 amino acid residues or more are not routinely prepared via stepwise solid phase synthesis. Synthesis of proteins and large polypeptides by this route is a time-consuming and laborious task. --

Page 14 has been amended as follows:

-- group of the N-terminal cysteine. Steps 2 and 3 can be repeated, as indicated by the arrow marked 4, for additional peptide segments. Also, a cleavable linker for purposes of monitoring the coupling and ligating reactions can be added between the "handle" and the "resin."

FIG. 22 is a reaction scheme for solid phase sequential ligation in the C- to N-terminal direction of PLA2G5.

FIG. 23 is a reaction scheme for synthesizing a Cam ester derivative for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 24 is a reaction scheme for synthesizing the C-terminal peptide segment for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 25A, B, and C is a diagram of a scheme for synthesizing an assembled polypeptide via bidirectional solid phase sequential ligation of two or more peptide segments.

FIG 26[A and B] are HPLC chromatographs following the solid phase solid phase native chemical ligation of 3 peptide segments in the N- to C- terminal direction, resulting in the assembled peptide, C5a 1-74.

FIG. 27 is a reaction scheme for synthesis of a C-terminal peptide segment for use in the solid phase native chemical ligations described herein, using a CAM ester cleavable handle to remove the synthesized peptide segment from the solid phase.

FIG. 28[A and B] are HPLC chromatographs and reconstructed ESI MS of the assembled peptide resulting from solid phase sequential ligation of 3 peptide segments: peptide segment 1 [(SEQ ID NO: 2)](CADRKNILA) (amino acids 19-27; SEQ ID NO:1), peptide segment 2 [(SEQ ID NO:3)] (CYGRLEEKKG) (amino acids 10-18; SEQ ID NO:1) and peptide segment 3 [(SEQ ID NO:4)] (ALTKYGFYGY) (amino acids 1-9; SEQ ID NO:1) on solid phase in the C- to N-terminal direction, using Fmoc protecting groups.

FIG. 29[A and B] are an HPLC chromatograph and ESI MS[, respectively,] of the final

ligation product, i.e. the first ligation product ligated to the third peptide segment (ALTKYGFYG) **(amino acids 1-9; SEQ ID NO:1)**, resulting from solid phase sequential ligation of 3 peptide segments in the C- to N-terminal direction, using ACM as the protecting group.

FIG. 30A-H are HPLC chromatographs and reconstructed ESI MS of the steps of synthesizing Phospholipase A2 Group 5, a 118 residue protein, using solid phase sequential native chemical ligation of four peptide segments in the C- to N-terminal direction. The first peptide segment is PLA2G5 88-118; the second is PLA2G5 59-87, the third is PLA2G5 26-58,

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Page 15 has been amended as follows:

-- and the fourth is PLA2G5 1-25. **FIG 30[32]A and B** are an HPLC chromatograph and reconstructed ESI MS of the first peptide segment, respectively. **FIG 30[32]C and D** are an HPLC chromatograph and reconstructed ESI MS, respectively, of the ligation product of the first and second peptide segments (PLA2G5 59-118). **FIG 30[32]E and F** are [an] an HPLC chromatograph and reconstructed ESI MS, respectively, of PLA2G5 26-118, the ligation product of PLA2G5 59-118 and PLA2G5 26-58 (the third peptide segment). **FIG 30[32]G and H** are HPLC chromatograph and reconstructed ESI MS, respectively, of PLA2G5 1-118, the assembled polypeptide.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Terminology

Amino acids: Amino acids include the 20 genetically coded amino acids, rare or unusual amino acids that are found in nature, and any of the non-naturally occurring and modified amino acids.

Aqueous solution: solutions containing water, including up to 8M urea in water, up to 6M guanidine- HCl in water, up to 60% acetonitrile in water.

Assembled Peptide: the final product of a solid phase sequential or bidirectional ligation, after cleavage of the cleavable handle, The assembled peptide comprises at least two separate peptide segments sequentially ligated on a solid phase. The assembled peptide may or may not have biological activity.

Cleavable Handle: A cleavable moiety that is capable of being selectively cleaved to release the assembled peptide from the solid phase. The cleavable handle must be capable of resisting cleavage under conditions suitable for coupling, activating, deprotecting, ligating, washing, and other

steps involved in the formation of an assembled peptide. The cleavable handle must also be stable to conditions used to produce the first peptide segment that is capable of being bound to a solid phase, including, for example, stepwise solid phase peptide synthesis. The cleavable handle preferably is located directly adjacent to the first peptide segment such that upon cleavage of the cleavable handle, the desired assembled peptide is released from the solid phase. The cleavable handle may be selected from any of the variety of cleavable handles used by those in the field. See, e.g., L. Canne et al., Tetrahedron Letters, 38(19):3361-3364 (1997); Ball et al., J. Pept. Sci, 1:288-294 (1995); Funakoshi et al, PNAS USA, 88:6981-6985 (1991); --

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Page 32 has been amended solely to introduce the phrase --(SEQ ID NO:2) -- at the last line of the depicted reaction schemes.

Table 1

Solid Phase Sequential Ligations: N- to C- Terminal	
3-Random Peptide Segment Model System	
<i>Lev-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS</i>) (1)	
+ <i>Resin-PCL-ONH₂</i>	
↓	1. pH 4.6, 6M GuHCl, 0.1 M acetate
<i>Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS</i>)	
(1)	
↓	2. pH 4.6, 6M GuHCl, 0.1 M acetate, 50 mM BrAcOH
<i>Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-</i>	
<i>COSAc</i> (1)	
↓	+ <i>H-CGFRVREFGDNTA-COS</i>) (2)
	3. pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol
<i>Resin-PCL-oxime-MSC-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG</i>	
↓	<i>CGFRVREF-GDNTA-COS</i>) (1+2)
	4. pH 4.6, 6M GuHCl, 0.1M acetate, 50mM BrAcOH
<i>Resin- PCL-oxime- MSC -LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG</i>	
	<i>CGFRVREF-GDNTA-COSAc</i> (1+2)
	+ <i>H-CADPSEEWVQKYVSDLELSA-OH</i> (3)
↓	5. pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol
<i>Resin- PCL-oxime- MSC -LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG</i>	
	<i>CGFRVREF-GDNTACADPSEEWVQKYVSDLELSA-OH</i> (1+2+3)
↓	6. pH 14, 6M GuHCl, 0.1M phosphate, 200mM hydrazine
<i>H-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHGCGFRVREF-</i>	
<i>GDNTACADPSEEWVQKYVSDLELSA-OH</i> (1+2+3) (SEQ ID NO:2)	
PCL= photocleavable linker	

Page 34 has been amended as follows:

reaction mixture is left standing at room temperature overnight. The next morning, the resin is washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample of resin are removed for monitoring by MALDI MS analysis.

The assembled peptide is removed from the solid phase via base cleavage of the cleavable handle from the remaining resin as outlined above only on a larger scale followed by purification by HPLC or desalting on PD-10 column and lyophilization.

Example 4: Solid Phase Native Chemical Ligation of C5a(1-74) (74aa) in the N- to C- Terminal Direction.

This example describes solid phase sequential native chemical ligation in the N- to C-terminal direction of C5a, Complement Factor 5A. The sequence of C5a is: [(SEQ ID NO. ?)]
TLQKKIEEEIAAKYKJSVVKCCYDGACVNDETCEQRAARISLGPKCIKAFTECCVVAS
QLRANISHKDMQLGR **(SEQ ID NO:3)**.

This peptide is prepared using solid phase sequential native ligation of 3 peptide segments: C5a(I-20), C5a(21-46), and C5a(47-74). The procedures used to synthesize C5a by solid phase ligations are identical to those described in the solid phase sequential native ligation of MIF (See Example 5).

Example 5: Solid Phase Sequential Native Chemical Ligation of MIF(1-115) (115 aa) in the N-Terminal to C- Terminal Direction.

The sequence of MIF(1-115) is [(SEQ.ID.NO.)]:

MPMFIVNTNVPRASVPDGFSEL TQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCAL
CSLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAASVGWNNSTFA **(SEQ ID NO:4)**

This peptide is prepared using solid phase sequential native ligation of 3 peptide segments: MIF(1-59) **(amino acid 1-59, SEQ ID NO:4)** MIF(60-80) **(amino acid 60-80, SEQ ID NO:4)** and MIF(81-115) **(amino acid 81-115, SEQ ID NO:4)**. See FIG. 16-20.

Page 36 has been amended as follows:

-- Example 6: Solid Phase Native Chemical. Ligation of Phospholipase A2, group 5(1-118) (118aa) in the C- to N-terminal Direction.

The sequence of Phospholipase A2, group 5 (PLA2G5) is: [(SEQ ID NO:)]

GLLDLKSMIEKVTGKNALTNYGFGYGCYCGWGGRGTPKDGTDWCCWAHDHCYGRLEE
KGCNIRTQSYKYRFAWGVVTCEPGPFCHVNLCACDRKLVYCLKRNLRSYNPQYQYFPN
ILCS (SEQ ID NO:5).

This peptide is prepared using solid phase sequential native ligation of 4 peptide segments: PLA2G5 (1-25), PLA2G5 (26-58), PLA2G5(59-87) and PLA2G5 (88-118). The procedures used to synthesize PLA2G5 by solid phase ligations are identical to those used for synthesizing the random sequence using ACM protection of the N-terminal Cys residues of the middle segments, as described in Example 9. See FIG. 22 for the reaction scheme. The Cam ester derivative is synthesized and incorporated into the C-terminal peptide segment according to the diagrams in FIG. 23, 24/FIG. 27. The assembled polypeptide, PLA2G5 (1-118), was folded and assayed for biological activity. It had the full activity of a recombinantly expressed PLA2G5.

Example 7: Preparation of Modified C-terminal Peptide Segment (on-resin CAM linker synthesis) (FIG. 27)

The commercial resin of choice (MBHA, any Boc-AA-OCH₂-Pam resin) is swelled in DMF

-TFA (1 min x 2) (not necessary if working with MBHA resin)

-DMF flow wash (30 sec x 2)

-addition of activated Boc-Lys(Fmoc)-OH (HBTU/DIEA activation), check for completion of reaction after 10-15 minutes by ninhydrin test

-DMF flow wash (30 sec x 2)

-TFA (1 min x 2) --

Page 38 has been amended as follows:

-- Example 8: Solid Phase Native Chemical Ligation of Random Peptide Segments in the C- to N-terminal Direction using Fmoc protection (See FIG. 28)

The following procedures can be used for solid phase ligations in the C- to N-terminal direction, as diagramed in Table 2. By example, a random peptide of [(SEQ ID NO: ?)]:

ALTKYGFYGCYGRLEEKGCADRKNILA (SEQ ID NO:1) can be ligated in three peptide segments (from C- to N-terminal direction): segment 1= CADRKNILA (amino acids 19-27; SEQ ID NO:1); segment 2 = CYGRLEEKGC (amino acids 10-18; SEQ ID NO:1); and segment 3 = ALTKYGFYGC (amino acids 1-9; SEQ ID NO:1).

The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. The modified C-terminal peptide segment (first peptide segment) is dissolved in 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (5 mM first peptide segment) and added to the resin and is left standing at room temperature overnight. The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample is removed for base cleavage and is treated with 8M urea, 0.1M NaPi, pH 7, treated for 2 minutes with 0.25N NaOH in the same 8M urea buffer (resulting pH~14), washed with an equal amount of 0.25N HCl in the same 8M urea buffer (resulting pH~2), and the combined eluants treated with TCEP prior to injection on HPLC.

In preparation for addition of the next segment, the resin is washed with 6M Gu•HCL, 0.1M NaPi, pH 7.0 (1 ml x 5) and drained. The second peptide segment (Fmoc-Cys-peptide-COSR) is dissolved in 6M Gu•HCL, 0.1M NaPi, pH 7.0, 0.5% thiophenol (to at least 10 mM to 50 mM second peptide segment) and added to the resin. [the] **The** mixture is left standing at room temperature overnight. The resin is washed with 6M Gu•HCL, 0.1M NaPi, pH 7.0 (1 ml x 5), water (1 ml x 5), DMF (1 ml x 5), and the Fmoc protecting group removed by treating with two aliquots of 20% piperidine in DMF (5 min each). The resin is then washed with DMF (1 ml x 5), water (1 --

Page 42 has been amended as follows: -- Table 2

<p>Polymer-Supported Ligations C- to N- Terminal Direction Fmoc Protection</p>	
	<p><i>H</i>-CADRKNILA-CAM-Lys(Levulinic acid)-NH₂ (1)</p> <p>+ Resin- ONH₂</p> <p>↓ 1. pH 4.6, 6M Gu•HCl, 0.1 acetate</p> <p><i>H</i>-CADRKNILA -CAM- Lys-oxime-Resin (1)</p> <p>+ <i>Fmoc</i>-CYGRLEEKG-COSR (2)</p> <p>↓ 2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol</p> <p><i>Fmoc</i>-CYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)</p> <p>↓ 3. 20% piperidine/DMF</p> <p><i>H</i>-CYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)</p> <p>+ <i>H</i>-ALTKYGFYG-COSR (3)</p> <p>↓ 4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol</p> <p><i>H</i>-ALTKYGFYGCYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2+3)</p> <p>↓ 5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH</p> <p><i>H</i>- ALTKYGFYGCYGRLEEKGCADRKNILA-OH (SEQ ID NO:1) --</p>

Page 43 has been amended to read: -- Table 3

Polymer-Supported Ligations

C- to N- Terminal Direction

ACM Protection

H-CADRKNILA-CAM-Lys(*Levulinic acid*)-NH₂ (1)

+ Resin- ONH₂

↓ 1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H-CADRKNILA -CAM-Lys-oxime-Resin (1)

+ *H*-C(ACM)YGRLEEKG-COSR (2)

↓ 2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-C(ACM)YGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)

↓ 3. a. mercury(II)acetate in 3% Aq. AcOH
b. 20% mercaptoethanol in pH 7.5, 6M Gu•HCl, 0.1 M phosphate

H-CYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)

+ *H*-ALTKYGFYG-COSR (3)

↓ 4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H- ALTKYGFYGCYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2+3)

↓ 5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYGCYGRLEEKGCADRKNILA-OH (SEQ ID NO:1) --

Figure 30 has been amended to include Figure sub-legends A-H.

In the Claims:

- 23. **[Amended]** A kit for preparing assembled polypeptides comprising:
- (a) a first [unprotected peptide segment, comprising] **container containing a solid support, having bound thereto a first partially or completely unprotected peptide having an N-terminus and a thioacid or a thioester of the formula –COSR at its C-terminus and [N-terminus], wherein said [first] partially or completely unprotected peptide [segment] is bound to [a] said solid [phase via a linker comprising a cleavable moiety] support via a linker; wherein said linker comprises a cleavable moiety and said partially or completely unprotected first peptide segment is bound to said linker at said N-terminus, and wherein R is a straight or branched C₁₋₁₅ functionalized alkyl group, a C₁₋₁₅ aromatic structure, or 1 to 4 amino acids or derivatives thereof;**
- (b) **a second container containing** a set of second **partially or completely** unprotected peptides [segments], each comprising a **thioester or a thioacid at its [their C-termini] C-terminus** and a cysteine **residue at its [their N-termini] N-terminus**; wherein [each] **the N-terminal cysteine** of said second [unprotected] peptide [segments have the same number of amino acids] **segment is capable of selectively ligating to the C-**

terminus of said solid phase-bound first peptide to form a solid phase-bound peptide comprising a thioacid at its C-terminus;
and

- (c) one or more **additional containers each containing a** set[s] of different **partially or completely** unprotected peptide segments, each comprising a thioester **or a thioacid** at **its** [their C-termini] **C-terminus** and a cysteine residue at **its** [their N-termini] **N-terminus**, wherein each of the members of each set have the same number of amino acids. --

24. [Amended] The kit of [claim 12] claim 23, wherein said set of second unprotected peptide segments **(b)** is comprised of peptides having the same length, but different amino acid sequences.
25. [Amended] The kit of [claim 12] claim 23, wherein said set of second unprotected peptides **(b)** consists essentially of identical peptides.
26. [Amended] The kit of [claim 13] **claim 23**, wherein said one or more sets of different unprotected peptides **(c)** comprise at least one set of peptides having the same length but different amino acid sequences.
29. [New] The kit of claim 23, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.
30. [New] The kit of claim 23, wherein the C-terminus of said first partially or completely unprotected peptide comprises said thioester of formula COSR.
31. [New] The kit of claim 23, wherein the C-terminus of said second partially or completely unprotected peptide comprises a thioacid.

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32. [New] The kit of claim 23, wherein the C-terminus of said second partially or completely unprotected peptide comprises said thioester of formula COSR.
33. [New] The kit of claim 23, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.
34. [New] The kit of claim 23, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.
35. [New] The kit of claim 23, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.
36. [New] The kit of claim 23, wherein said solid phase is a bead resin.
37. [New] The kit of claim 23, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.
38. [New] The kit of claim 23, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.
39. [New] The kit of claim 23, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.
40. [New] The kit of claim 23, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.
41. [New] A kit for preparing assembled polypeptides comprising:
a) a solid phase support having bound thereto a partially or completely unprotected first peptide segment comprising an N-terminus and a C-terminus, wherein said peptide is bound to said

support via a cleavable linkage between C-terminus and said support, and wherein said N-terminus is a cysteine residue.

- b) a set of second partially or completely unprotected peptides, each comprising a thioester of formula COSR or a thioacid at its C-terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said solid phase-bound first peptide segment is capable of selectively ligating to the C-terminus of said second peptide to form a solid phase-bound peptide comprising a cysteine at its N-terminus; wherein R is a straight or branched C₁₋₁₅ functionalized alkyl group, a C₁₋₁₅ aromatic structure, or 1 to 4 amino acids or derivatives thereof; and
- c) one or more sets of different partially or completely unprotected peptide segments, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each of the members of each set have the same number of amino acids. --

42. [New] The kit of claim 41, wherein said set of second unprotected peptide segments is comprised of peptides having the same length, but different amino acid sequences.
43. [New] The kit of claim 41, wherein said set of second unprotected peptides consists essentially of identical peptides.
44. [New] The kit of claim 41, wherein said one or more sets of different unprotected peptides (c) comprise at least one set of peptides having the same length but different amino acid sequences.
45. [New] The kit of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.

46. **[New]** The kit of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises said thioester of formula COSR.
47. **[New]** The kit of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises a thioacid.
48. **[New]** The kit of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises said thioester of formula COSR.
49. **[New]** The kit of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.
50. **[New]** The kit of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.
51. **[New]** The kit of claim 41, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.
52. **[New]** The kit of claim 41, wherein said solid phase is a bead resin.
53. **[New]** The kit of claim 41, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.
54. **[New]** The kit of claim 41, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.
55. **[New]** The kit of claim 41, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.

56. [New] The kit of claim 41, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.

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Appendix B: The Pending Claims

To facilitate the Examiner's review of the patentability of the present invention, Applicant has reproduced below the presently pending claims.

23. **[Amended]** A kit for preparing assembled polypeptides comprising:
- (a) a first container containing a solid support, having bound thereto a first partially or completely unprotected peptide having an N-terminus and a thioacid or a thioester of the formula $-\text{COSR}$ at its C-terminus and, wherein said partially or completely unprotected peptide is bound to said solid support via a linker; wherein said linker comprises a cleavable moiety and said partially or completely unprotected first peptide segment is bound to said linker at said N-terminus, and wherein R is a straight or branched C_{1-15} functionalized alkyl group, a C_{1-15} aromatic structure, or 1 to 4 amino acids or derivatives thereof;
 - (b) a second container containing a set of second partially or completely unprotected peptides, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said second peptide segment is capable of selectively ligating to the C-terminus of said solid phase-bound first peptide to form a solid phase-bound peptide comprising a thioacid at its C-terminus; and
 - (c) one or more additional containers each containing a set of different partially or completely unprotected peptide segments, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each of the members of each set have the same number of amino acids.

24. **[Amended]** The kit of claim 23, wherein said set of second unprotected peptide segments (b) is comprised of peptides having the same length, but different amino acid sequences.
25. **[Amended]** The kit of claim 23, wherein said set of second unprotected peptides (b) consists essentially of identical peptides.
26. **[Amended]** The kit of claim 23, wherein said one or more sets of different unprotected peptides (c) comprise at least one set of peptides having the same length but different amino acid sequences.
29. **[New]** The kit of claim 23, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.
30. **[New]** The kit of claim 23, wherein the C-terminus of said first partially or completely unprotected peptide comprises said thioester of formula COSR.
31. **[New]** The kit of claim 23, wherein the C-terminus of said second partially or completely unprotected peptide comprises a thioacid.
32. **[New]** The kit of claim 23, wherein the C-terminus of said second partially or completely unprotected peptide comprises said thioester of formula COSR.
33. **[New]** The kit of claim 23, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.
34. **[New]** The kit of claim 23, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.

35. [New] The kit of claim 23, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.
36. [New] The kit of claim 23, wherein said solid phase is a bead resin.
37. [New] The kit of claim 23, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.
38. [New] The kit of claim 23, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.
39. [New] The kit of claim 23, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.
40. [New] The kit of claim 23, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.
41. [New] A kit for preparing assembled polypeptides comprising:
 - a) a solid phase support having bound thereto a partially or completely unprotected first peptide segment comprising an N-terminus and a C-terminus, wherein said peptide is bound to said support via a cleavable linkage between C-terminus and said support, and wherein said N-terminus is a cysteine residue.
 - b) a set of second partially or completely unprotected peptides, each comprising a thioester of formula COSR or a thioacid at its C-terminus and a cysteine residue at its N-terminus; wherein the N-terminal cysteine of said solid phase-bound first peptide segment is capable of selectively ligating to the C-terminus of said second peptide to form a solid phase-bound peptide comprising a cysteine at its N-terminus; wherein R is a straight or branched C₁₋₁₅

functionalized alkyl group, a C₁₋₁₅ aromatic structure, or 1 to 4 amino acids or derivatives thereof; and

- c) one or more sets of different partially or completely unprotected peptide segments, each comprising a thioester or a thioacid at its C-terminus and a cysteine residue at its N-terminus, wherein each of the members of each set have the same number of amino acids. --

42. **[New]** The kit of claim 41, wherein said set of second unprotected peptide segments is comprised of peptides having the same length, but different amino acid sequences.
43. **[New]** The kit of claim 41, wherein said set of second unprotected peptides consists essentially of identical peptides.
44. **[New]** The kit of claim 41, wherein said one or more sets of different unprotected peptides (c) comprise at least one set of peptides having the same length but different amino acid sequences.
45. **[New]** The kit of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises a thioacid.
46. **[New]** The kit of claim 41, wherein the C-terminus of said first partially or completely unprotected peptides (a) comprises said thioester of formula COSR.
47. **[New]** The kit of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises a thioacid.
48. **[New]** The kit of claim 41, wherein the C-terminus of said second partially or completely unprotected peptides (b) comprises said thioester of formula COSR.

49. [New] The kit of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises a thioacid.
50. [New] The kit of claim 41, wherein the C-terminus of at least one of said partially or completely unprotected peptides (c) comprises said thioester of formula COSR.
51. [New] The kit of claim 41, wherein said assembled polypeptide is from 20 to 1000 amino acids in length.
52. [New] The kit of claim 41, wherein said solid phase is a bead resin.
53. [New] The kit of claim 41, wherein said first, second and third peptide segments range in size from 5 to 99 amino acid residues.
54. [New] The kit of claim 41, wherein said first, second and third peptide segments are all prepared by solid phase synthesis.
55. [New] The kit of claim 41, wherein the last peptide segment to be ligated onto the solid phase-bound peptide is derived from recombinant DNA expression.
56. [New] The kit of claim 41, wherein at least one of said peptide segments (a), (b) or (c) comprises an unnatural backbone structure.

APPENDIX C

Year	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100
1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	

1 **SOLID PHASE NATIVE CHEMICAL LIGATION**
2 **OF UNPROTECTED OR N-TERMINAL CYSTEINE PROTECTED PEPTIDES**
3 **IN AQUEOUS SOLUTION**

4
5 CROSS-REFERENCE TO RELATED APPLICATIONS

6 This application is a continuation-in part of, and claims the benefit of, U.S. Provisional
7 Application No. 60/049,553, filed June 13, 1997, herein incorporated by reference, and is a
9 divisional application of, and claims the benefit of, U.S. Patent Application Serial No.
10 09/097,094, herein incorporated by reference.

11 Background

12 Existing methods for, the chemical synthesis of proteins include stepwise solid phase
13 synthesis, and fragment condensation either in solution or on solid phase. The classic stepwise
14 solid phase synthesis of Merrifield involves covalently linking an amino acid corresponding to
15 the carboxy-terminal amino acid of the desired peptide chain to a solid support and extending
16 the polypeptide chain toward the amino end by stepwise coupling of activated amino acid
17 derivatives having activated carboxyl groups. After completion of the assembly of the fully
18 protected solid phase bound peptide chain, the peptide-solid phase covalent attachment is
19 cleaved by suitable chemistry and the protecting groups removed to give the product
20 polypeptide.

21 Some disadvantages of the stepwise solid phase synthesis method include: incomplete
22 reaction at the coupling and deprotection steps in each cycle results in formation of solid-phase
23 bound by products. Similarly, side reactions due to imperfections in the chemistry, and or
24 impurities present in the reagents/protected amino acids, all lead to a multiplicity of solid phase
25 bound products at each step of the chain assembly and to the formation of complex product
26 mixtures in the final product. Thus, the longer the peptide chain, the more challenging it is to
27 obtain high-purity well-defined products. Due to the production of complex mixtures, the
28 stepwise solid phase synthesis approach has size limitations. In general, well-defined
29 polypeptides of 100 amino acid residues or more are not routinely prepared via stepwise solid
30 phase synthesis. Synthesis of proteins and large polypeptides by this route is a time-consuming
31 and laborious task.

group of the N-terminal cysteine. Steps 2 and 3 can be repeated, as indicated by the arrow marked 4, for additional peptide segments. Also, a cleavable linker for purposes of monitoring the coupling and ligating reactions can be added between the "handle" and the "resin."

FIG. 22 is a reaction scheme for solid phase sequential ligation in the C- to N-terminal direction of PLA2G5.

FIG. 23 is a reaction scheme for synthesizing a Cam ester derivative for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 24 is a reaction scheme for synthesizing the C-terminal peptide segment for solid phase sequential ligation in the C- to N-terminal direction.

FIG. 25A, B, and C is a diagram of a scheme for synthesizing an assembled polypeptide via bidirectional solid phase sequential ligation of two or more peptide segments.

FIG 26 are HPLC chromatographs following the solid phase solid phase native chemical ligation of 3 peptide segments in the N- to C- terminal direction, resulting in the assembled peptide, C5a 1-74.

FIG. 27 is a reaction scheme for synthesis of a C-terminal peptide segment for use in the solid phase native chemical ligations described herein, using a CAM ester cleavable handle to remove the synthesized peptide segment from the solid phase.

FIG. 28 are HPLC chromatographs and reconstructed ESI MS of the assembled peptide resulting from solid phase sequential ligation of 3 peptide segments: peptide segment 1 (CADRKNILA) (amino acids 19-27; SEQ ID NO:1), peptide segment 2 (CYGRLEEKKG) (amino acids 10-18; SEQ ID NO:1) and peptide segment 3 (ALTKYGFYGG) (amino acids 1-9; SEQ ID NO:1) on solid phase in the C- to N-terminal direction, using Fmoc protecting groups.

FIG. 29 are an HPLC chromatograph and ESI MS of the final ligation product, i.e. the first ligation product ligated to the third peptide segment (ALTKYGFYGG) (amino acids 1-9; SEQ ID NO:1), resulting from solid phase sequential ligation of 3 peptide segments in the C- to N-terminal direction, using ACM as the protecting group.

FIG. 30A-H are HPLC chromatographs and reconstructed ESI MS of the steps of synthesizing Phospholipase A2 Group 5, a 118 residue protein, using solid phase sequential native chemical ligation of four peptide segments in the C- to N-terminal direction. The first peptide segment is PLA2G5 88-118; the second is PLA2G5 59-87, the third is PLA2G5 26-58,

1 and the fourth is PLA2G5 1-25. **FIG 30A and B** are an HPLC chromatograph and reconstructed
2 ESI MS of the first peptide segment, respectively. **FIG 30C and D** are an HPLC chromatograph
3 and reconstructed ESI MS, respectively, of the ligation product of the first and second peptide
4 segments (PLA2G5 59-118). **FIG 30E and F** are an an HPLC chromatograph and reconstructed
5 ESI MS, respectively, of PLA2G5 26-118, the ligation product of PLA2G5 59-118 and PLA2G5
6 26-58 (the third peptide segment). **FIG 30G and H** are HPLC chromatograph and reconstructed
7 ESI MS, respectively, of PLA2G5 1-118, the assembled polypeptide.

8

9 DESCRIPTION OF SPECIFIC EMBODIMENTS

10 Terminology

11 Amino acids: Amino acids include the 20 genetically coded amino acids, rare or unusual
12 amino acids that are found in nature, and any of the non-naturally occurring and modified amino
13 acids.

14 Aqueous solution: solutions containing water, including up to 8M urea in water, up to 6M
15 guanidine- HCl in water, up to 60% acetonitrile in water.

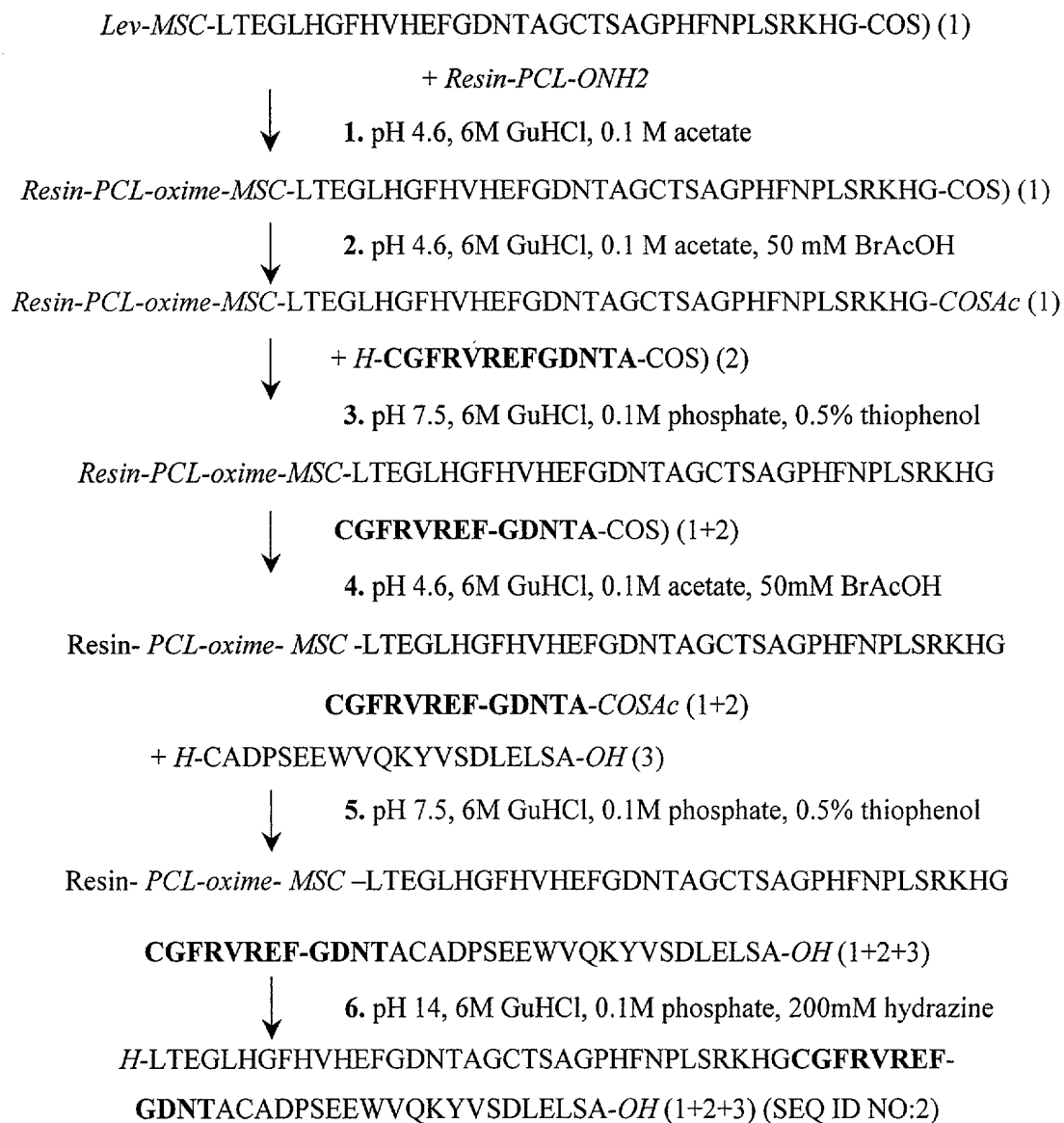
16 Assembled Peptide: the final product of a solid phase sequential or bidirectional ligation,
17 after cleavage of the cleavable handle, The assembled peptide comprises at least two separate
18 peptide segments sequentially ligated on a solid phase. The assembled peptide may or may not
19 have biological activity.

20 Cleavable Handle: A cleavable moiety that is capable of being selectively cleaved to
21 release the assembled peptide from the solid phase. The cleavable handle must be capable of
22 resisting cleavage under conditions suitable for coupling, activating, deprotecting, ligating,
23 washing, and other steps involved in the formation of an assembled peptide. The cleavable
24 handle must also be stable to conditions used to produce the first peptide segment that is capable
25 of being bound to a solid phase, including, for example, stepwise solid phase peptide synthesis.
26 The cleavable handle preferably is located directly adjacent to the first peptide segment such that
27 upon cleavage of the cleavable handle, the desired assembled peptide is released from the solid
28 phase. The cleavable handle may be selected from any of the variety of cleavable handles used
29 by those in the field. See, e.g., L. Canne et al., Tetrahedron Letters, 38(19):3361-3364 (1997);
30 Ball et al., J. Pept. Sci, 1:288-294 (1995); Funakoshi et al, PNAS USA, 88:6981-6985 (1991);

Table 1

Solid Phase Sequential Ligations: N- to C- Terminal

3-Random Peptide Segment Model System



PCL= photocleavable linker

1 reaction mixture is left standing at room temperature overnight. The next morning, the resin is
2 washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample of
3 resin are removed for monitoring by MALDI MS analysis.

4 The assembled peptide is removed from the solid phase via base cleavage of the
5 cleavable handle from the remaining resin as outlined above only on a larger scale followed by
6 purification by HPLC or desalting on PD-10 column and lyophilization.

7
8 **Example 4: Solid Phase Native Chemical Ligation of C5a(1-74) (74aa) in the N- to C-**
9 **Terminal Direction.**

10 This example describes solid phase sequential native chemical ligation in the N- to C-
11 terminal direction of C5a, Complement Factor 5A. The sequence of C5a is:

12 TLQKKIEEEIAAKYKJSVVKCCYDGACVNNDTCEQRAARISLGPKCIKAFTECCVVAS
13 QLRANISHKDMQLGR (SEQ ID NO:3).

14 This peptide is prepared using solid phase sequential native ligation of 3 peptide
15 segments: C5a(1-20), C5a(21-46), and C5a(47-74). The procedures used to synthesize C5a by
16 solid phase ligations are identical to those described in the solid phase sequential native ligation
17 of MIF (See Example 5).

18 **Example 5: Solid Phase Sequential Native Chemical Ligation of MIF(1-115) (115 aa) in the**
19 **N- Terminal to C- Terminal Direction.**

20 The sequence of MIF(1-115) is:

21 MPMFIVNTNVPRASVPDGLSELTQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCAL
22 CSLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAASVGWNNSTFA
23 (SEQ ID NO:4)

24 This peptide is prepared using solid phase sequential native ligation of 3 peptide segments:

25 MIF(1-59) (amino acid 1-59, SEQ ID NO:4) MIF(60-80) (amino acid 60-80, SEQ ID NO:4) and
26 MIF(81-115) (amino acid 81-115, SEQ ID NO:4). See FIG. 16-20.

1 **Example 6: Solid Phase Native Chemical. Ligation of Phospholipase A2, group 5(1-118)**
2 **(118aa) in the C- to N-terminal Direction.**

3
4 The sequence of Phospholipase A2, group 5 (PLA2G5) is:

5 GLLDLKSMIEKVTGKNALTNYGFGYGCYCGWGGRGTPKDGTDWCCWAHDHCYGRLEE
6 KGCNIRTQSYKYRFAWGVVTCEPGPFCHVNLACDRKLVYCLKRNLRSYNPQYQYFPN
7 ILCS (SEQ ID NO:5).

8
9 This peptide is prepared using solid phase sequential native ligation of 4 peptide segments:
10 PLA2G5 (1-25), PLA2G5 (26-58), PLA2G5(59-87) and PLA2G5 (88-118). The procedures
11 used to synthesize PLA2G5 by solid phase ligations are identical to those used for synthesizing
12 the random sequence using ACM protection of the N-terminal Cys residues of the middle
13 segments, as described in Example 9. See **FIG. 22** for the reaction scheme. The Cam ester
14 derivative is synthesized and incorporated into the C-terminal peptide segment according to the
15 diagrams in **FIG. 23, 24/FIG. 27**. The assembled polypeptide, PLA2G5 (1-118), was folded and
16 assayed for biological activity. It had the full activity of a recombinantly expressed PLA2G5.

17
18 **Example 7: Preparation of Modified C-terminal Peptide Segment (on-resin CAM linker**
19 **synthesis) (FIG. 27)**

20 The commercial resin of choice (MBHA, any Boc-AA-OCH₂-Pam resin) is swelled in DMF
21 -TFA (1 min x 2) (not necessary if working with MBHA resin)
22 -DMF flow wash (30 sec x 2)
23 -addition of activated Boc-Lys(Fmoc)-OH (HBTU/DIEA activation), check for completion of
24 reaction after 10-15 minutes by ninhydrin test
25 -DMF flow wash (30 sec x 2)
26 -TFA (1 min x 2) --

Example 8: Solid Phase Native Chemical Ligation of Random Peptide Segments in the C- to N-terminal Direction using Fmoc protection (See FIG. 28)

The following procedures can be used for solid phase ligations in the C- to N-terminal direction, as diagramed in Table 2. By example, a random peptide of:

ALTKYGFYGCYGRLEEKGCAADRKNILA (SEQ ID NO:1) can be ligated in three peptide segments (from C- to N-terminal direction): segment 1= CADRKNILA (amino acids 19-27; SEQ ID NO:1); segment 2 = CYGRLEEKG (amino acids 10-18; SEQ ID NO:1); and segment 3 = ALTKYGFYG (amino acids 1-9; SEQ ID NO:1).

The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. The modified C-terminal peptide segment (first peptide segment) is dissolved in 6MGu•HCL, 0.1M Na Acetate, pH 4.6 (5 mM first peptide segment) and added to the resin and is left standing at room temperature overnight. The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample is removed for base cleavage and is treated with 8M urea, 0.1M NaPi, pH 7, treated for 2 minutes with 0.25N NaOH in the same 8M urea buffer (resulting pH~14), washed with an equal amount of 0.25N HCl in the same 8M urea buffer (resulting pH~2), and the combined eluants treated with TCEP prior to injection on HPLC.

In preparation for addition of the next segment, the resin is washed with 6M Gu•HCL, 0.1M NaPi, pH 7.0 (1 ml x 5) and drained. The second peptide segment (Fmoc-Cys-peptide-COSR) is dissolved in 6M Gu•HCL, 0.1M NaPi, pH 7.0, 0.5% thiophenol (to at least 10 mM to 50 mM second peptide segment) and added to the resin. The mixture is left standing at room temperature overnight. The resin is washed with 6M Gu•HCL, 0.1M NaPi, pH 7.0 (1 ml x 5), water (1 ml x 5), DMF (1 ml x 5), and the Fmoc protecting group removed by treating with two aliquots of 20% piperidine in DMF (5 min each). The resin is then washed with DMF (1 ml x 5), water (1

Table 2

Polymer-Supported Ligations

C- to N- Terminal Direction

Fmoc Protection

H-CADRKNILA-CAM-Lys(Levulinic acid)-NH₂ (1)

+ Resin- ONH₂



1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H-CADRKNILA -CAM- Lys-oxime-Resin (1)

+ Fmoc-CYGRLEEKG-COSR (2)



2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

Fmoc-CYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)



3. 20% piperidine/DMF

H-CYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)

+ *H*-ALTKYGFYG-COSR (3)



4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-ALTKYGFYGCYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2+3)



5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYGCYGRLEEKGCADRKNILA-OH (SEQ ID NO:1)

Polymer-Supported Ligations

C- to N- Terminal Direction

ACM Protection

H-CADRKNILA-CAM-Lys(Levulinic acid)-NH₂ (1)

+ Resin- ONH₂



1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H-CADRKNILA -CAM-Lys-oxime-Resin (1)

+ *H*-C(ACM)**YGRLEEK**G-COSR (2)



2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-C(ACM)**YGRLEEK**GCADRKNILA-CAM-Lys-oxime-Resin (1+2)



3. a. mercury(II)acetate in 3% Aq. AcOH
b. 20% mercaptoethanol in pH 7.5, 6M Gu•HCl, 0.1 M phosphate

H-**CYGRLEEK**GCADRKNILA-CAM-Lys-oxime-Resin (1+2)

+ *H*-ALTKYGFY**G**-COSR (3)



4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-ALTKYGFY**G****CYGRLEEK**GCADRKNILA-CAM-Lys-oxime-Resin (1+2+3)



5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H-ALTKYGFY**G****CYGRLEEK**GCADRKNILA-OH (SEQ ID NO:1)